**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| In re application of: | : |
| Deborah O'Neil | : |
| | : Group Art Unit: |
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| | : Examiner: Necholus Ogden, Jr |
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| For: Anti-Microbial Compositions | : ONEIL-0002US |

**DECLARATION OF DEBORAH O'NEIL PURSUANT TO 37 C.F.R. § 1.132**

I, Deborah O'Neil, hereby declare as follows:

1. I am the named inventor for the above-mentioned application.
2. Under my direction, two studies were undertaken by Dr. Stanley E. Katz: Study 1 to show the anti-microbial effects of *Ranunculus* extracts and Study 2 to determine the antibiotic resistance development caused by *Ranunculus* extracts.
3. The procedures for the studies are as outlined in Ward et al. Journ. Food Protection 65(3):528-533 (2002) (Appendix A).
4. *Staphylococcus aureus* (*S. aureus*) ATCC 29213 and *Escherichia coli* (*E. coli*) ATCC 25922 were chosen for use as gram positive and gram negative indicator organisms in Study 1. Both organisms exhibit a reasonably wide spectrum of sensitivity to antibiotics and anti-microbials.
5. Tryptic soy agar (TSA) was used for the agar diffusion assays. Tryptic soy broth (TSB) was used as the growth media for the Minimal Inhibitory Concentration (MIC) determinations.
6. The culture stocks were prepared by the following procedure. First, lyophilized organisms were inoculated into TSB and incubated overnight at $37^0$C with shaking. The overnight growth of organisms was streaked for isolation onto TSA plates, and incubated overnight at $37^0$C. Individual colonies were picked and incubated overnight at $37^0$C with shaking in TSB to provide test strains. Test strains were stored on TSA slants at $4^0$C. The strains were transferred to fresh slants weekly. For each study fresh overnight cultures were prepared.

7. In Study 1, *Ranunculus* extracts were prepared from: dry leaves, dry whole plant, frozen whole plant, roots and shoots. Each of these were separately extracted with 50% and 95% ethanol. Following the extraction, 200 µl of each extract was added to 3 ml of medium (TSB).

8. The agar diffusion assay is widely used to determine the dose response of an organism to an antibiotic or another substance. Extracts and dilutions of a wide variety of materials may be added to cylinders placed on top of organism-seeded agar or into wells cut in the seed agar. After incubation, the inhibitory activity is estimated by the size of the circular zones of inhibition, which are proportional to the inhibitory activity and concentration of the inhibitory material in the assay well.

9. The standard agar diffusion assay as used here involved a seeded TSA medium: 20 ml of seeded TSA (cooled to $50^0$C before inoculation with $10^5$ colony forming units (CFU)/ml of indicator organism) was added to a petri dish. Extract material was placed into cylinders (six total cylinders) placed on the surface of the agar seeded with the organism. A separate seeded plate was dedicated to each product and each organism. Each well of the plate was charged with 100 µl of test material. Plates were incubated overnight at $37^0$C. After an overnight incubation, the edge of the growth was sliced, removed and placed into TSB for growth. This growth is defined as Passage 1. This actively growing passage was then diluted and placed into TSA for exposure to the test material to generate Passage 2. The same procedure was repeated to generate Passage 3. Study 1 was designed to determine the anti-microbial effects of the extracted plant materials.

10. Tables 1 and 2 (Appendix B) show the results of Study 1 (Table 1 displays the *S. aureus* results and Table 2 displays the *E. coli* results). The results are given as the diameter of the zone of inhibition for each sample, in millimeters. As can be seen from Tables 1-2, *Ranunculus* extracts exhibited significant anti-microbial effects. It was determined that the 50% ethanol extracts of *Ranunculus* were the best extracts to study the effect of the antibiotic resistance development.

11. In Study 2, samples were prepared by extracting the following parts of the *Ranunculus* plant with 50 ml of 50% ethanol: whole plant, 7.5g; whole winter

dried, 10g; Root bulbs, 10g; fresh tops, 5g, salve 1, 5 g; salve 2, 5 g. Following extraction, 200 µl of each extract was added to 3 ml of medium (TSB). Additionally, 25 µl and 50 µl of the root bulb extract were added to 3 ml of medium to generate a dose response titration.

12. The MIC clinical method was used to evaluate the potential of *Ranunculus* extracts to affect the antibiotic susceptibility of the indicator organisms. This methodology is usually used in a liquid (so called planktonic or free growth) rather than a solid medium. The amount growth yields an approximate value for the antibiotic resistance of the assay microorganisms.

13. The panel of antibiotics used in the MIC assay as markers of resistance were ampicillin, streptomycin, oxytetracycline, erythromycin, gentamycin, and norfloxacin. These antibiotics were chosen as a reasonable spectrum of currently used antibiotics.

14. Generally, a sensitive organism is added to a nutrient broth at a level that approximates $10^3$ organisms/ml medium. A known volume of medium is added to the first well and the contents mixed. An equal volume of the antibiotic is added to the first well and the contents mixed, followed by the transfer of a known volume of the first well to the second well. The volume transferred is usually the volume of the inoculated broth placed initially into the well. By repeating the transfer a titration series is created. For example, if the concentration of the antibiotic added to the first well is 1024 µg/ml, after addition the well will contain an antibiotic concentration of 512 µg/ml. Repeating this dilution, the titration series will be 256, 128, 64, 32, 16, 8, 4, 2, 1 µg/ml and so on. The endpoint or MIC is defined as the last well where there is no growth.

15. The MIC baselines for the *S. aureus* test panel were as follows: ampicillin, 2.0 µg/ml; streptomycin, 8.0 µg/ml; oxytetracycline, 0.125 µg/ml; erythromycin, 0.125 µg/ml; gentamycin, 0.5 µg/ml; and norfloxacin, 0.8 µg/ml.

16. The MIC baselines for the *E. coli* test panel were as follows: ampicillin, 1.0 μg/ml; streptomycin, 4.0 μg/ml; oxytetracycline, 0.125 μg/ml; erythromycin, 0.125 μg/ml; gentamycin, 0.5 μg/ml; and norfloxacin, 0.8 μg/ml.

17. For the MIC test procedure used herein, overnight cultures of the indicator organisms were diluted to approximately $10^5$ CFU/ ml. An approximate volume of the $10^5$ CFU dilution was added to sterile TSB to give an approximately $10^3$ CFU/ml level of organism. Each of the wells of a 96-well microtiter plate was charged with 125 μl of the seeded TSB using a multi-channel pipetter. To the first well of the row, 125 μl of the *Ranunculus* extract was added and mixed thoroughly. After mixing, 125 μl of the well contents were transferred to the next well in the row. This process was repeated for 11 of the 12 wells. The last well served as a growth control. A sterile reagent blank was also used in an identical fashion to ensure that reagent effects, if present, were not misinterpreted.

18. After an overnight incubation at $37^0$C, the microtiter plates where placed in an enzyme-linked immunosorbant assay reader (SLT Lab Instruments, Research Triangle Park, NC) and the absorbance of each well measured at 620 nm. The MIC was defined as the concentration of the last well where no growth occurred. The growth differential for estimating the MIC was an absorbance increase of at least twice the baseline absorbance.

19. Tables 3-12 (Appendix C) display the MIC test results. Tables 3 and 4 are the test results for *E. coli* and *S. aureus*, respectfully, as tested against whole plant extract. In Study 2, after each passage, indicator organisms were isolated for MIC determination and comparison with unexposed indicator organisms.

20. Tables 5-6 display the test results from whole/winter dried extract. Tables 7-8 display the test data from the root bulb extract at a 200 μl extract/ 3mL medium concentration, the first two passages were skipped because it appeared the organism population was not stabilized as far as resistance development. The

organisms for this were taken from the edge of very large zones of inhibition and may not have had time to develop resistance because of the "kill" concentration. Hence, the possible lack of resistance selection. For the *S. aureus*, zones in the diffusion assay plates were large but organism collection from the edges was possible.

21. Table 9 displays an attempt to use a dose response titration, a dosage of 25 µl extract/ 3 ml medium. Again using the indicator *E. coli*, passage 1 and 2 organisms were not used. *S. aureus* was not tested.

22. Tables 10-11 display the third concentration (50 µl extract/ 3 ml medium ) of root bulb extract used. This third concentration of root bulb extract yielded no changes in baseline MIC values with the exception of norfloxacin, where a substantive increase was noted. The third passage was deemed superfluous as the pattern with previous concentrations was already seen. For the *S. aureus* indicator, only passage 3 organisms were used. They showed the same substantive increases in resistance across all the antimicrobial families of the panel.

23. Table 12 displays the data from testing fresh tops at 200 µl extract/ 3 ml medium. Only the *E. coli* was used for resistance studies because no zones of inhibition were noted for *S. aureus* in the screening assay. 26. Thus as can be seen from Study 1, the *Ranuculus* plant extracts display anti-microbial activity against both gram-positive and gram-negative organisms. Additionally, certain extracts have the added and unexpected benefit of inducing resistance of certain organisms to antibiotics slowly if at all.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that the statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By: _____
Deborah O'Neil

Dated: 1/6/05

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APPENDIX A

# Inhibition, Resistance Development, and Increased Antibiotic and Antimicrobial Resistance Caused by Nutraceuticals

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## ABSTRACT

With the increasing use of herbal remedies by the general public worldwide, there remains a lack of information on the relationship between nutraceutical use and antibiotic resistance. Historically, there have been claims that nutraceuticals possess antibacterial and antiviral activity. However, the claims come with little or no documentation and no information related to the development of resistance to the nutraceutical or the cause of increases in resistance to antibiotics. These studies investigate the ability of nutraceutical exposure to influence the development of antibiotic resistance in bacteria. Two antibiotic-sensitive organisms, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922, were used as representative of the gram-positive and gram-negative bacteria. These preliminary investigations showed a general increase to the ampicillin marker by the products studied, using *Staphylococcus aureus* ATCC 29213 as the indicator organism. There were 13 product-related increases in the MIC, 2 decreases, and 7 no changes. All six of the garlic products increased the MIC of the norfloxacin marker to greater than fourfold above baseline. Using *E. coli* ATCC 25922 as the indicator organism, the greatest product–antibiotic marker interaction was with the ampicillin marker. Garlic, *Echinacea*, and zinc products all caused large increases in the MIC to ampicillin over baseline values.

Nutraceuticals and most dietary supplements fall under the Nutrition Labeling and Education Act of 1990 and are unregulated by the Food and Drug Administration. Nutraceuticals were described by Adelaja et al. *(1)* as "naturally occurring or enhanced foods produced and purchased with the belief that the product will promote health or confer a medicinal benefit, including short- or long-term prevention or treatment of disease, whether or not the anticipated benefit is claimed or substantiated." The value of the nutraceuticals market is estimated by the Nutrition Business Journal *(2)* to be $80 billion in the United States alone; approximately 60 million people use herbal products *(11)*. These products are consumed daily by large numbers of people and also used widely for animals worldwide, without medical advice or supervision.

People are attracted to the use of nutraceuticals for a number of reasons: (i) the all-too-common adverse effects related to pharmaceuticals, (ii) the high cost of prescription pharmaceuticals, (iii) the reluctance of some insurance providers to cover the costs of drugs, and (iv) the perception that natural is good. There have been both peer-reviewed research and media attempts to enlighten consumers to the potential dangers of the unrestricted use of natural agents *(4, 10, 14)*. As more consumers add nutraceuticals to their pharmacopoeia of disease preventatives and/or remedies, the call for research into the danger of combining seemingly harmless nutraceuticals with prescription drugs increases. Reports of the interactions of nutraceuticals with prescription drugs have become commonplace *(5, 13)*. Unfortunately, nutraceuticals are nonstandardized within product type, have variable dosages, and, all too often, lack standardized materials for dose-response titrations.

For many years, nonregulated dietary supplements or nutraceuticals have been studied, primarily outside the United States. There is a large compendium of work on topics ranging from specific and nonspecific cellular immune function, characterization of enzyme activities, treatments for specific disease states, and others *(12)*. There is also a dearth of information related to the development of bacterial resistance to the nutraceutical and any nutraceutical effects on the development of antibacterial resistance. There is a lack of information available on how nutraceuticals play a role in the inhibition of microorganisms and alteration of bacterial populations, particularly in the gastrointestinal tract and/or on skin. Extensive laboratory research and clinical trials are necessary to develop a database for physicians' recommendations to patients. Unfortunately, the Dietary Supplement Health and Education Act of 1994 places the burden of proof that a dietary supplement is unsafe on the government.

This research report examines a subtle effect of some nutraceuticals by surveying the ability of a relatively small sampling of commercially available nutraceuticals to interact with indicator microorganisms to enhance, diminish, or have no effect on the susceptibility or resistance of microorganisms. Many nutraceuticals carry with them (although not on the label) suggestions of antimicrobial properties *(6, 12, 13, 15–17)*. Compounds not suspected of having any

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antibacterial properties, such as pesticides, were shown to increase the MICs of antibiotics against a strain of *Staphylococcus aureus* (ATCC 9144) both alone and in conjunction with antibiotics, all at legal food residue levels *(3)*. Hence, the significance of statements and implications that many nutraceuticals will cure or ameliorate a wide variety of human ailments requires an investigation of the effects of a variety of nutraceuticals on indicator microorganisms to determine if the organisms develop resistance to the nutraceutical and if there is also an increase in resistance to common antibiotics.

## MATERIALS AND METHODS

**Nutraceutical products and manufacturers.** The following is a list of the nutraceutial products and their manufacturers: *Aloe vera*, Nature's Herbs Inner Leaf Brand Aloe Vera (Twinlabs, American Fork, Utah), Nature's Way Brand Aloelax Herbal Stimulant Laxative (Nature's Way Products Inc., Springville, Utah); *Echinacea* sp., Nature's Herbs Brand Time Release (Twinlabs), Nature's Way Brand Optimized Extract Echina Guard (Nature's Way Products), Nature's Herbs Brand Echinacea Root and Herb (Twinlabs), Nature's Way Brand Echina-Guard Liquid (Nature's Way Products); *Allium sativum*, Schiff Brand Super Garlic Concentrate (Schiff Products Inc., Salt Lake City, Utah), Quintessence Brand Standardized Maximum Allicin Garlic Caplets (Natrol Inc. Chatsworth, Calif.), Enzymatic Therapy Brand Garlinase (Enzymatic Therapy, Green Bay, Wis.), Nature's Way Brand Garlicin Tablets (Nature's Way Products), Sunsource Brand Garlique Tablets (Chatlem Inc., Chatlanooga, Tenn.), Natrol Brand Garlipure (Natrol); *Hydrastis canadensis*, Nature's Way Brand Wild American Golden Seal Herb (Nature's Way Products), Nature's Herbs Wild Countryside Brand Golden Seal Root (Nature's Herbs-Twinlabs), Nature's Answer Brand Golden Seal Root (Nature's Herbs-Twinlabs); *Hypericum perforatum*, Nature's Plus Herbal Actives Brand St. John's wort (Nature's Plus, Natural Organics Inc., Melville, N.Y.), Nature's Herbs Power-herbs Brand St. John's wort (Twinlabs), Enzymatic Therapy Natural Medicines Brand St. John's wort (Enzymatic Therapy), Nature's Way Brand St. John's wort (Nature's Way Products), GAIA Herbs Brand St. Johnswort Supreme (Gaia Herbs Inc., Brevard, N.C.); zinc, GNC Cherry Flavor Zinc Lozenges with Vitamin C (GNC Corp., Pittsburgh, Pa.), Cold-Eeze Citrus Flavor Zinc Gluconate Lozenges (Quigley Corporation, Doylestown, Pa.).

**Indicator organisms.** The organisms used were *S. aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 and were chosen as representatives of gram-positive and gram-negative organisms. Both organisms exhibited a reasonably wide spectrum of sensitivity to antibiotics and antimicrobials. The panel of antibiotics used as markers of resistance were ampicillin, streptomycin, oxytetracycline, erythromycin, gentamycin, and norfloxacin. These antibiotics and antimicrobials represent a reasonable spectrum of the antibiotic and antibacterial families commonly used.

The MICs for *S. aureus* ATCC 29213 for this panel in μg/ml were as follows: ampicillin, 2.0; streptomycin, 8.0; oxytetracycline, 0.125; erythromycin, 0.125; gentamycin, 0.5; and norfloxacin, 0.8. For *E. coli* ATCC 25922, the MIC values in μg/ml were as follows: ampicillin, 1.0; streptomycin, 4.0, oxytetracycline, 0.125; erythromycin, 0.25; gentamycin, 0.125; and norfloxacin, 0.8. Both indicator organisms reflected very similar sensitivities to the test panel.

**Culture preparation.** Tryptic soy broth (TSB) and tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) were used as the growth media. TSB was used for all MIC determinations and TSA was used for the diffusion assays.

Culture stocks were prepared by inoculating lyophilized *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 into TSB and incubating at 37°C overnight with shaking. The organisms were streaked for isolation onto TSA plates and incubated overnight at 37°C. Individual colonies were picked and inoculated into TSB and grown as described. Storage of the test strains was on TSA slants at 4°C. The organisms were transferred to fresh slants biweekly. Before any studies, a fresh overnight culture was prepared.

TABLE 1. *Individual, daily, and manufacturer s aavertisea equivalents for nutraceutical products*

| Product | Recommended dose (g) | | Dosage label protency (mg/d) | Equivalent (mg/ml/d)[a] |
|---|---|---|---|---|
| | Single | Daily | | |
| *Aloe vera* 1 | 0.65 | 0.65 | 250 | 5 |
| *Aloe vera* 2 | 0.62 | 0.62 | 250 | 5 |
| Goldenseal 1 | 0.98 | 3.92 | 3,200 | 64 |
| Goldenseal 2 | 1.32 | 3.96 | 3,240 | 64.8 |
| Goldenseal 3 | 0.33 | 0.99 | 990 | 19.8 |
| St. John's wort 1 | 0.93 | 1.21 | 900 | 18 |
| St. John's wort 2 | 0.51 | 1.53 | 900 | 18 |
| St. John's wort 3 | 0.38 | 1.14 | 900 | 18 |
| St. John's wort 4 | 1.12 | 2.24 | 1,800 | 36 |
| St. John's wort 5 | 1.0 | 4.0 | 4,000 | 80 |
| Garlic 1 | 1.54 | 1.54 | 16,850 | 337 |
| Garlic 2 | 0.83 | 1.66 | 1,200 | 24 |
| Garlic 3 | 0.47 | 0.47 | 320 | 6.4 |
| Garlic 4 | 0.88 | 1.76 | 518 | 10.4 |
| Garlic 5 | 0.83 | 0.83 | 400 | 8 |
| Garlic 6 | 1.21 | 2.42 | 2,000 | 40 |
| *Echinacea* 1 | 1.63 | 1.63 | 750 | 15 |
| *Echinacea* 2 | 1.08 | 4.32 | 29,600 | 592 |
| *Echinacea* 3 | 1.08 | 4.23 | 3,636 | 72.7 |
| *Echinacea* 4 | 1.66 | 5,01 | 5,010 | 100.2 |
| Zinc 1 | 2.83 | 8.39 | 60 | 1.2 |
| Zinc 2 | 4.53 | 27.36 | 80 | 1.6 |

[a] Equivalent diluted in 50 ml of 50% ethanol as a tincture.

**Extraction of nutraceuticals from pill and capsule forms.** The initial and daily dose product label recommendations for dry products either in capsule or tablet form were weighed (Table 1). Pills and opened capsules were individually immersed in flasks containing 50 ml of 50% ethanol and extracted at room temperature using a shaker at 200 rpm for 24 h. Commercially produced tinctures were used directly from the package. Controls were adjusted to reflect ethanol concentrations in either the laboratory-extracted or manufacturer-packaged tinctures. Tinctures and extract supernatants were filter sterilized using a 0.2-μm filter (Gelman Sciences, Ann Arbor, Mich.) into sterile screw-cap test tubes and stored at room temperature.

**Determination of MICs.** The basic analytical system to evaluate the potential of nutraceuticals to affect the antibiotic or antimicrobial susceptibility of the indicator microorganisms was the MIC clinical method *(8)*. Twofold dilution sequences of extracts or tinctures were used in the MIC determinations.

Overnight cultures of the listed ATCC microorganisms were grown in TSB. Based on turbidity measurements, correlated with plate counts, the cultures were diluted to approximately $10^5$ CFU/

ml. An appropriate volume of the approximately $10^5$-CFU/ml dilution was added to sterile TSB to give an approximately $10^3$-CFU/ml level of organism. Each of the wells of a 96-well microtiter plate was charged with 125 µl of seeded TSB using a multichannel pipetter. To the first well of a row, 125 µl of the nutraceutical extract was added and mixed well. After mixing, 125 µl of the well contents was transferred to the next well. This transfer procedure continued for 11 of the 12 wells. The last well acted as a growth control. Along with each extract being screened, a sterile reagent alcohol blank was used in an identical fashion to ensure that reagent effects, if present, were not misinterpreted.

After an overnight incubation at 37°C, the microtiter plates were placed into an enzyme-linked immunosorbent assay reader (SLT Lab instruments, Research Triangle Park, N.C.) and the absorbance of each well measured at 620 nm. The MIC was defined as the concentration of the last well where no growth occurred. When working with natural product material, there may be a less defined MIC end point; the pertinent growth differential for estimating the MIC was an absorbance increase of at least twice the baseline absorbance.

**Agar diffusion assay.** The agar-well diffusion assay is widely used to determine the dose response of an organism to an antibiotic or unknown inhibitory substance *(7, 9)*. Extracts and dilutions of a wide variety of materials, such as fermentation broths, synthetic organics, and plant and soil extracts, are added to cylinders placed atop organism-seeded agar or in wells cut into the seeded agar. After incubation, the inhibitory activity is estimated by the size of the circular zones of inhibition, which is proportional to the concentration of the inhibitory material in the assay well.

In the adaptation of the agar diffusion system used herein, 20 ml of seeded TSA medium (cooled to 50°C before inoculation with $10^5$ CFU/ml of indicator organism) was added to the petri dish. When the seeded agar solidified, six evenly spaced, 8-mm wells were cut in a circular fashion into each plate. A separate seeded plate was dedicated to each product. Each well of the plate was charged with 100 µl; well 1 contained the full-strength tincture, wells 2 through 5 were charged each with 100 µl of an aliquot of the decreasing 1:1 dilution series of the full-strength extract, and well 6 was charged with the diluent, as a control. Plates were incubated overnight at 37°C. The petri plates were removed from the incubator and both clear zones of inhibition and zones showing regrowth were measured using a Fisher-Lilly Zone Reader (Fisher Scientific Company, Suwanee, Ga.).

To examine the development of resistance after exposure to the nutraceutical extract, areas where colonies appeared in the agar of the clear zones of inhibition or where organism regrowth was apparent were removed asceptically and the organisms grown in TSB. These organisms were then used as the next inoculum for the diffusion assay to examine the development of resistance. Repeating this process after each exposure allowed for an extended view of resistance development.

## RESULTS AND DISCUSSION

The choice of which nutraceuticals to study for possible effects on antibacterial activity and/or effects on antibiotic activity was somewhat daunting, considering the choices of products available on the shelves of health food stores, large multifunctional retail outlets, pharmacies, and supermarkets. In these studies, 22 products representing six different "active" ingredients were studied for both their antibacterial activity and what effects these materials had on the MICs of the test organisms. The six different products were *Aloe*, goldenseal, garlic, St. John's wort, *Echinacea*, and zinc gluconate and zinc oxide. Along with the active ingredients, there may be other constituents, such as vitamins and flavorings, that might possess "activities." *Aloe* was reported to possess antiseptic properties; goldenseal was reported to have weak antiseptic properties; garlic was reported to have antimicrobial activity; St. John's wort was used to treat gastritis; *Echinacea* was used as an antiinfective and to treat hard-to-heal wounds, although touted to be an immune system stimulant; and, zinc, in the oxide and chelated form, was purported to shorten the duration of the common cold *(12)*.

TABLE 2. *Resistance development in Staphylococcus aureus ATCC 29213 caused by nutraceutical alcohol extracts*

| Product representative | Product extract dilution per activity (%)[a] | | | | |
|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 |
| *Aloe vera* | 30 | 14 | 10 | —[b] | — |
| Goldenseal | 15 | 7 | 4 | — | — |
| St. John's wort | 5 | — | — | — | — |
| Zinc | 30[c] | — | — | — | — |

[a] Data are percentage of activity after two passages.
[b] No zones of inhibition.
[c] Estimated value; insufficient points on response line.

Table 1 presents a summary of the products, label recommendations, and dosages used in the study. This table presents both the problem and dilemma of nutraceutical products. The daily doses are very variable and may be functions of the "potency" of the product. However, since potency does not seem to be standardized with any degree of accuracy or even against standard active materials, it becomes very difficult to compare potencies and effects. The *Aloe* and zinc products present essentially identical dosages; the St. John's wort product dosages varied approximately 4.5-fold; the garlic products varied approximately 52.6-fold; the *Echinacea* dosages varied approximately 39.4-fold; and the goldenseal products varied approximately 3.3-fold. Based on the product dosages, correlations with any responses were essentially impossible.

To establish a baseline of whether any of these products possessed antimicrobial activities, two analytical procedures were used, the agar-well plate diffusion procedure and the MIC. The agar-well technique was used to determine whether the extract and/or its dilutions had any antibacterial activity; the second analytical system, the MIC, was used to determine the presence of antibacterial activity in the extracts and titrate such activity. In both cases, activity was estimated as units related to the extract rather than against any defined standard. Unfortunately, there are no authoritative standards for the active ingredient in many of these products and little information related to antibacterial activities.

Table 2 shows the agar diffusion responses for a representative of the *Aloe vera*, goldenseal, St. John's wort, and zinc products. The arbitrary values, as a percentage of the product dosage, were a function of the extract concen-

tration: 100, 50, 25, 12.5, and 6.25%, respectively. Measurable activity (log of concentration versus diameter of zone of inhibition) was found for some of the extracts using both the *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 test strains. Organisms isolated from within the agar of the relatively clear zones of inhibition or areas of regrowth indicated that the original assay organism was exhibiting resistance to the extract. This type of resistance was not necessarily related to resistance to antibiotics or antimicrobials used in human or veterinary medicine but indicates only that the materials studied showed decreased inhibitory properties against specific organisms. Garlic and *Echinacea* products did not show inhibitory activity in the MIC screening and therefore were not included in the agar diffusion studies. In general, resistance to the product extracts occurred rapidly, as demonstrated by much smaller or no zones of inhibition after one to two exposures. Using *S. aureus* ATCC 29213, the *Aloe* product's inhibitory response decreased after one passage and reached a constant level. However, the two lowest dilutions showed no inhibitory activity after a single passage; also, the inhibitory activity decreased to approximately 30% of the original level. The levels of inhibitory activity of the goldenseal product decreased to approximately 15% of the original antibacterial activity after a single exposure, with the lowest three concentrations essentially showing either traces of or no measurable activity. The St. John's wort product extract also produced a significant increase in resistance (decrease in inhibitory zone size) in the *S. aureus* ATCC 29213 after one passage (exposure), a loss of inhibitory activity of approximately 95%. Similarly, the response of the zinc product extracts paralleled that of the St. John's wort in that inhibitory activity decreased very quickly. Against *E. coli* ATCC 29522, only three product extracts, two goldenseal and one St. John's wort, showed initial inhibitory activity. However, after one passage, the organisms isolated from the zones of inhibition showed no inhibitory response. As occurred with *S. aureus* ATCC 29213, a decrease in inhibitory activity or resistance to the nutraceutical develops rather quickly.

Baseline MIC antibiotic sensitivities of the two indicator organisms were compiled by two investigators working totally independently and confirming the values obtained by each other. These values were the basis of any conclusions related to increases, decreases, or no effects of the extracts of the products. It is not uncommon for MIC values to vary between half and twice the expected MIC value (this is equivalent to one well on either side). This is considered to be normal variation around the MIC. Before any value would be considered as a significant or substantive change (not statistical), an MIC value would have to be either four times greater or four times less than the established value. It is quite inappropriate to apply strict statistical analyses to this analytical system *(4, 8)*.

Table 3 shows the results of the exposure of *S. aureus* ATCC 29213 to both the extract and the antibiotic marker panel. Against the oxytetracycline marker, there was only one product, an *Echinacea* (*Echinacea* sp. 3), that showed any response, an increase in the MIC to oxytetracycline

TABLE 3. *MICs (μg/ml) of antimicrobials resulting from nutraceutical extract and antimicrobial using* Staphylococcus aureus *ATCC 29213 as the indicator organism*[a]

| Product | OTC | AMP | ERY | STR | GEN | NOR |
|---|---|---|---|---|---|---|
| Baseline value | 0.125 | 2.0 | 0.125 | 8.0 | 0.5 | 0.8 |
| *Aloe vera* 1 | 0.125 | 4.0 | 0.125 | 8.0 | 1.0 | 3.2 |
| *Aloe vera* 2 | 0.125 | 32.0 | 0.25 | 4.0 | 1.0 | 3.2 |
| Goldenseal 1 | 0.25 | 4.0 | 0.25 | 4.0 | 1.0 | 1.6 |
| Goldenseal 2 | 0.125 | 4.0 | 0.25 | 4.0 | 1.0 | 3.2 |
| Goldenseal 3 | 0.125 | 0.5 | 0.25 | 0.5 | 0.5 | 0.4 |
| St. John's wort 1 | 0.125 | 4.0 | 0.25 | 0.5 | 0.5 | 0.4 |
| St. John's wort 2 | 0.125 | 8.0 | 0.125 | 4.0 | 1.0 | 1.6 |
| St. John's wort 3 | 0.125 | 2.0 | 0.25 | 4.0 | 1.0 | 1.6 |
| St. John's wort 4 | 0.125 | 4.0 | 0.25 | 4.0 | 0.5 | 1.6 |
| St. John's wort 5 | 0.125 | 0.25 | 0.125 | 0.125 | 1.0 | 0.1 |
| Garlic 1 | 0.125 | 64.0 | 0.25 | 8.0 | 1.0 | 3.2 |
| Garlic 2 | 0.125 | 64.0 | 0.25 | 4.0 | 1.0 | 3.2 |
| Garlic 3 | 0.125 | 32.0 | 0.25 | 4.0 | 1.0 | 3.2 |
| Garlic 4 | 0.125 | 32.0 | 0.25 | 4.0 | 1.0 | 3.2 |
| Garlic 5 | 0.125 | 16.0 | 0.25 | 2.0 | 0.5 | 3.2 |
| Garlic 6 | 0.125 | 64.0 | 0.25 | 4.0 | 1.0 | 3.2 |
| *Echinacea* 1 | 0.25 | 32.0 | 0.50 | 4.0 | 1.0 | 3.2 |
| *Echinacea* 2 | 0.50 | 64.0 | 0.50 | 8.0 | 1.0 | 3.2 |
| *Echinacea* 3 | 0.50 | 32.0 | 0.50 | 8.0 | 2.0 | 3.2 |
| *Echinacea* 4 | 0.25 | 64.0 | 0.50 | 8.0 | 2.0 | 6.4 |
| Zinc 1 | 0.125 | 4.0 | 0.25 | 4.0 | 1.0 | 1.6 |
| Zinc 2 | 0.125 | 8.0 | 0.25 | 4.0 | 1.0 | 1.6 |

[a] OTC, oxytetracycline; AMP, ampicillin; ERY, erythromycin; STR, streptomycin; GEN, gentamycin; and NOR, norfloxacin.

from 0.125 to 0.50 μg/ml of oxytetracycline. The responses for the ampicillin marker were, predominantly, increases greater or equal to fourfold in 13 MIC values, decreases caused by two products, and no changes in seven. In the two *Aloe* extracts, one product caused a 16-fold increase and one affected no significant change. Two of the three goldenseal products (*H. canadensis* 1 and 2) caused no significant changes in MIC, whereas one, *H. canadensis* 3, caused a significant decrease. In the five samples of St. John's wort, there was one significant or substantive increase and one significant or substantive decrease in MIC. All six samples of the garlic products caused significant or substantive increases in MIC; the extracts of all four samples of *Echinacea* also increased significantly the MIC to the ampicillin marker. Only one of the two zinc products significantly increased the MIC to ampicillin. There were no significant increases or decreases in MIC caused by any of the product extracts to erythromycin. Against the streptomycin marker, there were no significant or substantive increases in MIC; two product extracts, a goldenseal (*H. canadensis* 3) and a St. John's wort extract (*H. perforatum* 4), affected decreases in MIC greater than fourfold. The two *Echinacea* extracts (*Echinacea* sp. 4 and 5) showed fourfold increases in the MIC to gentamycin. Both extracts of *Aloe* caused fourfold increases in MIC to norfloxacin as did one of the goldenseal extracts. One of the five extracts of St. John's wort caused a greater than fourfold decrease in MIC against the norfloxacin marker, whereas all six of the garlic product extracts increased the MIC greater than

TABLE 4. *MICs (μg/ml of antimicrobial) resulting from nutraceutical extract and antimicrobial using Escherichia coli ATCC 25922 as the indicator organism*[a]

| Product | OTC | AMP | ERY | STR | GEN | NOR |
|---|---|---|---|---|---|---|
| Baseline value | 0.125 | 1.0 | 0.25 | 4.0 | 0.125 | 0.8 |
| *Aloe vera* 1 | 0.125 | 0.125 | 0.125 | 2.0 | 0.125 | 1.6 |
| *Aloe vera* 2 | 0.25 | 8.0 | 0.125 | 4.0 | 0.125 | 1.6 |
| Goldenseal 1 | 0.063 | 0.125 | 0.063 | 0.5 | 0.063 | 0.4 |
| Goldenseal 2 | 0.25 | 0.50 | 0.125 | 2.0 | 0.125 | 0.8 |
| Goldenseal 3 | 0.125 | 0.25 | 0.063 | 0.25 | 0.125 | 0.1 |
| St. John's wort 1 | 0.063 | 0.125 | 0.063 | 0.125 | 0.063 | 0.4 |
| St. John's wort 2 | 0.125 | 2.0 | 0.125 | 2.0 | 0.125 | 0.4 |
| St. John's wort 3 | 0.063 | 1.0 | 0.063 | 0.25 | 0.125 | 0.4 |
| St. John's wort 4 | 0.063 | 2.0 | 0.125 | 1.0 | 0.125 | 0.8 |
| St. John's wort 5 | 0.063 | 0.125 | 0.125 | 0.125 | 0.063 | 0.063 |
| Garlic 1 | 0.125 | 2.0 | 0.125 | 4.0 | 0.125 | 1.6 |
| Garlic 2 | 0.125 | 8.0 | 0.125 | 2.0 | 0.125 | 1.6 |
| Garlic 3 | 0.125 | 32.0 | 0.125 | 4.0 | 0.25 | 1.6 |
| Garlic 4 | 0.125 | 16.0 | 0.125 | 4.0 | 0.125 | 1.6 |
| Garlic 5 | 0.125 | 16.0 | 0.125 | 2.0 | 0.063 | 1.6 |
| Garlic 6 | 0.125 | 16.0 | 0.125 | 2.0 | 0.125 | 1.6 |
| *Echinacea* 1 | 0.125 | 16.0 | 0.125 | 2.0 | 0.125 | 1.6 |
| *Echinacea* 2 | 0.125 | 16.0 | 0.125 | 2.0 | 0.063 | 0.8 |
| *Echinacea* 3 | 0.063 | 16.0 | 0.125 | 4.0 | 0.125 | 0.1 |
| *Echinacea* 4 | 0.125 | 64.0 | 0.125 | 4.0 | 0.125 | 1.6 |
| Zinc 1 | 0.063 | 8.0 | 0.125 | 2.0 | 0.063 | 1.6 |
| Zinc 2 | 0.063 | 16.0 | 0.125 | 2.0 | 0.125 | 1.6 |

[a] OTC, oxytetracycline; AMP, ampicillin; ERY, erythromycin; STR, streptomycin; GEN, gentamycin; and NOR, norfloxacin.

fourfold. Three of the four *Echinacea* extracts showed significant or substantive increases in MIC, whereas none of the zinc product extracts affected the MIC to norfloxacin.

Table 4 shows the effects of the nutraceutical extracts on the MIC of the six marker antibiotics against *E. coli* ATCC 25922. There were no beyond-limits changes in the MIC values with both the oxytetracycline and gentamycin markers. Against the ampicillin marker, one *Aloe* product (*A. vera* 1), two goldenseal products (*H. canadensis* 1 and 3), and two St. John's wort products (*H. perforatum* 1 and 5) caused decreases in MIC of greater than fourfold against the ampicillin marker; in contrast, five of six garlic extracts, all four *Echinacea* extracts, and both zinc product extracts increased the MIC to ampicillin. Two goldenseal (*H. canadensis* 1 and 3) and two St. John's wort extracts (*H. perforatum* 1 and 3) decreased the MICs to the erythromycin marker. Decreased MICs to streptomycin appeared with two of three goldenseal (*H. canadensis* 1 and 3), and three of the five St. John's wort extracts; none of the other extracts affected the MIC values. Single decreases in the MIC to norfloxacin were noted in three products, goldenseal (*H. canadensis* 3), St. John's wort (*H. perforatum* 5), and *Echinacea* (*Echinacea* sp. 3). However, there was no consistency of product and resistance response with this marker. The greatest consistency of product and marker appeared with ampicillin wherein garlic, *Echinacea*, and zinc product extracts all caused very large increases in MIC values. There was a trend for decreases in MIC with goldenseal to the ampicillin, erythromycin, and streptomycin markers.

It is not the decreases in MIC that are worrisome but the fact that extracts of some products, garlic, *Echinacea*, and zinc, caused substantial increases in the MIC to the ampicillin marker with both the gram-positive and gram-negative indicator organisms. Ampicillin, one of the β-lactam antibiotics, remains one of the more commonly used drugs to fight a wide variety of infective medical problems. Similarly, the increases in the MIC of the *S. aureus* ATCC 29213 found in the presence of *Aloe*, garlic, and *Echinacea* extracts pose similar worries. Although there is no direct evidence in the literature that any of these products have compromised therapy, the fact that there are increases in the MIC (resistance) related to some products requires considerably more study. Considering the numbers and variety of products in the market place, more extensive investigations are required.

What are the implications of this resistance development beyond the fact that some members of this small sampling of nutraceuticals can be selective agents in the development of antibiotic resistance and that the products in this small sample lose their antibacterial properties quickly. These implications are conjectural at this time, but point to what may be a major problem with those nutraceuticals purported to have antibacterial properties. These antibacterial properties, if they exist outside in vitro studies, might be very transitory with resistance to the active ingredient in the nutraceutical building quickly. Hence, effectiveness is at best short term and at worse not effective. This leaves the consumer with a potentially worse situation for using the nutraceutical. The development of resistance to antibiotics can pose even greater long-term problems. If an infection occurs from bacteria exposed to the nutraceuticals, it could be more refractory to treatment. This could pose

significant physical hardship on any individual so afflicted and an increased financial burden. There is no doubt that these implications must be investigated further.

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## APPENDIX B

Study 1 Anti-Microbial Effects

Table 1. *S. aureus*

| Passage | 1 | 1 | 2 | 2 | 3 | 3 |
|---|---|---|---|---|---|---|
| Alcohol Extract | 50% | 95% | 50% | 95% | 50% | 95% |
| Dry Leaf | 19 mm | 100 mm | 15 mm | 12 mm | 15 mm | 15 mm |
| Dry Plant | 22 mm | 26 mm | 20 mm | 20 mm | 16 mm | 16 mm |
| Frozen | 0 mm | 0 mm | 0 mm | 0 mm | 0 mm | 0 mm |
| Roots | 100 mm | 100 mm | 100 mm | 100 mm | 10 mm | 100 mm |
| Shoots | 15 mm | 100 mm | 12 mm | 100 mm | 0 mm | 25 mm |

Table 2. *E. coli*

| Passage | 1 | 1 | 2 | 2 | 3 | 3 |
|---|---|---|---|---|---|---|
| Alcohol Extract | 50% | 95% | 50% | 95% | 50% | 95% |
| Dry Leaf | 20 mm | 100 mm | 20 mm | 15 mm | 13 mm | 10 mm |
| Dry Plant | 100 mm | 100 mm | 100 mm | 100 mm | 20 mm | 100 mm |
| Frozen | 0 mm | 0 mm | 0 mm | 0 mm | 0 mm | 0 mm |
| Roots | 100 mm | 100 mm | 100 mm | 100 mm | 10 mm | 100 mm |
| Shoots | 15 mm | 100 mm | 12 mm | 100 mm | 0 mm | 25 mm |

## APPENDIX C

Study 2: MIC Results

Table 3. *Ranunculus* Whole Plant Extract
Planktonic Dosage: 200 µl /3 ml

| Organism *E. coli* | | | | |
|---|---|---|---|---|
| | Control | Passage 1 | Passage 2 | Passage 3 |
| MIC Standards, µg/ml | | | | |
| Oxytetracycline | 2 | 4 | 2 | 4 |
| Ampicillin | 16 | 32 | 64 | 64 |
| Erythromycin | 32 | 64 | 32 | 32 |
| Neomycin | 16 | 32 | 64 | 64 |
| Streptomycin | 8 | 64 | 32 | 64 |
| Norfloxacin | .05 | 1.6 | 0.8 | 1.6 |

Table 4. *Ranunculus* Whole Plant Extract
Planktonic Dosage: 200 µl /3 ml

| Organism *S. aureus* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| oxytetracycline | 0.5 | 0.5 | 0.125 | 0.25 |
| Ampicillin | .0125 | 0.125 | 0.063 | 0.125 |
| Erythromycin | 0.25 | 0.25 | 0.063 | 0.125 |
| Neomycin | 4 | 4 | 1 | 1 |
| Streptomycin | 8 | 8 | 4 | 4 |
| Norfloxacin | 0.8 | 0.8 | 0.8 | 0.8 |

Table 5. *Ranunculus* Whole Plant Extract-Winter Dried
Planktonic Dosage: 200 µl /3 ml

| Organism *E. coli* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| oxytetracycline | 2 | 2 | 4 | 2 |
| Ampicillin | 16 | >16 | >16 | 8 |
| Erythromycin | 32 | 32 | 32 | 32 |
| Neomycin | 16 | 64 | 32 | 16 |
| Streptomycin | 8 | 64 | 32 | 16 |
| Norfloxacin | 0.05 | 0.8 | 0.8 | *0.2 |

Table 6. *Ranunculus* Whole Plant Extract-Winter Dried
Planktonic Dosage: 200 µl /3 ml

| Organism *S. aureus* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| Oxytetracycline | 0.5 | 0.25 | 0.25 | 1 |
| Ampicillin | .0125 | 0.125 | 0.25 | 16 |
| Erythromycin | 0.25 | 0.25 | 0.125 | 32 |
| Neomycin | 4 | 4 | 2 | 8 |
| Streptomycin | 8 | 8 | 8 | 8 |
| Norfloxacin | 0.8 | 0.8 | 1.6 | 0.8 |

Table 7. *Ranunculus* Root Bulb
Planktonic Dosage: 200 µl /3 ml

| Organism *E. coli* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| Oxytetracycline | 2 | -- | -- | 0.5 |
| Ampicillin | 16 | -- | -- | 8 |
| Erythromycin | 32 | -- | -- | 32 |
| Neomycin | 16 | -- | -- | 32 |
| Streptomycin | 8 | -- | -- | 8 |
| Norfloxacin | 0.05 | -- | -- | 0.1 |

Table 8. *Ranunculus* Root Bulb
Planktonic Dosage: 200 µl /3 ml

| Organism *S. aureus* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/µl | | | | |
| Oxytetracycline | 0.5 | 64 | 64 | 64 |
| Ampicillin | 0.125 | 64 | 64 | 64 |
| Erythromycin | 0.25 | 64 | 64 | 64 |
| Neomycin | 4 | 64 | 64 | 64 |
| Streptomycin | 8 | 64 | 64 | 64 |
| Norfloxacin | 0.8 | 51.2 | 51.2 | 51.2 |

Table 9. *Ranunculus* Root Bulb
Planktonic Dosage: 25 µl /3 ml

| Organism *E. coli* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| Oxytetracycline | 2 | -- | -- | 1 |
| Ampicillin | 16 | -- | -- | 8 |
| Erythromycin | 32 | -- | -- | 32 |
| Neomycin | 16 | -- | -- | 16 |
| Streptomycin | 8 | -- | -- | 16 |
| Norfloxacin | 0.05 | -- | -- | 0.2 |

Table 10. *Ranunculus* Root Bulb
Planktonic Dosage: 50 µl /3 ml

| Organism *E. coli* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| Oxytetracycline | 2 | 2 | 1 | -- |
| Ampicillin | 16 | 8 | 16 | -- |
| Erythromycin | 32 | 32 | 32 | -- |
| Neomycin | 16 | 8 | 16 | -- |
| Streptomycin | 8 | 16 | 8 | -- |
| Norfloxacin | 0.05 | 1.6 | 0.2 | -- |

Table 11. *Ranunculus* Root Bulb
Planktonic Dosage: 50 µl /3 ml

| Organism *S. aureus* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/µl | | | | |
| Oxytetracycline | .05 | -- | -- | 64 |
| Ampicillin | 0.125 | -- | -- | 64 |
| Erythromycin | 0.25 | -- | -- | 64 |
| Neomycin | 4 | -- | -- | 64 |
| Streptomycin | 8 | -- | -- | 64 |
| Norfloxacin | 0.8 | -- | -- | 51.2 |

Table 12. *Ranunculus* Fresh Tops
Planktonic Dosage: 200 µl /3 ml

| Organism *E. coli* | | | | |
|---|---|---|---|---|
| | Control | Pass 1 | Pass 2 | Pass 3 |
| MIC Standards, µg/ml | | | | |
| Oxytetracycline | 2 | 2 | 8 | 8 |
| Ampicillin | 16 | <1 | <1 | <1 |
| Erythromycin | 32 | 32 | 32 | 32 |
| Neomycin | 16 | 1 | 32 | 64 |
| Streptomycin | 8 | 32 | 32 | 64 |
| Norfloxacin | 0.05 | 0.8 | 1 | 1 |